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Hsp70 suppresses apoptosis of BRL cells by regulating the expression of Bcl-2, cytochrome C, and caspase 8/3

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Abstract During cold stress, liver cells undergo apoptotic injury as a result of oxidative stress. Heat shock 70 kDa protein (Hsp70) is a protein involved in modulating a variety of physiological processes, including stress responses, proliferation, and apoptosis. In addition, Hsp70 regulates apoptotic signaling pathways in different manners, promoting or suppressing apoptosis. In this study, we investigated the effects of Hsp70 overexpression on hydrogen peroxide (H_2O_2)-induced apoptosis of Buffalo rat liver (BRL) cells and the underlying mechanisms of these effects. Our results show that in comparison with the control group, Hsp70 overexpression displayed increased protein levels of Bcl-2, and decreased cytochrome c (Cyt *c*), cleaved caspase 3, and cleaved caspase 8, but no apparent differences were found in levels of Bax. Furthermore, Hsp70 overexpression significantly suppresses the amount of apoptotic cells. Such findings indicate that overexpression of Hsp70 inhibits H_2O_2-mediated activation of caspase 8 and caspase 3, upregulates the expression of Bcl-2 which is a known anti-apoptotic protein, and decreases the release of Cyt *c* from the mitochondria into the cytoplasm, collectively decreasing cell apoptosis.

Keywords Hsp70 · Apoptosis · Oxidative stress

Introduction

Cold stress affects animal growth, development, and health, which can ultimately harm livestock husbandry. The mechanisms of response to cold stress are complicated; almost all organs and tissues are involved. It has been demonstrated that cold stress causes relatively severe damage to the liver due to decreased levels of glutathione (GSH) and increased levels of protein carbonylation, lipid oxidation, and abundance of

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intracellular reactive oxygen species (ROS), which collectively leads to oxidative stress-induced apoptosis in the liver (Sahin and Gumuslu 2004, 2007).

Apoptosis can be triggered by a variety of factors, among which ROS-induced oxidative stress is a key step (Ryter *et al.* 2007). For example, it has been reported that low concentration of hydrogen peroxide (H_2O_2) can induce apoptosis (Davies 1999; Troyano *et al.* 2003). The mechanisms of apoptosis are complex, as relevant signaling pathways communicate with one another to collectively regulate the life and death of cells. In death signaling pathways, CD95 (Fas) and tumor necrosis factor (TNF) family proteins cleave and activate downstream target proteins by activating caspase 8, eventually leading to apoptosis (Cohen 1997). In mitochondrial pathways, cytochrome C (Cyt *c*) released from the mitochondria binds to apoptosis-activating factor 1 (Apaf-1) and activates the caspase 9/caspase 3 cascade. In addition, Cyt *c* also changes the ratio of apoptosis-related proteins Bcl-2/Bax, thereby triggering apoptosis (Perkins *et al.* 2000).

Heat shock 70 kDa protein (Hsp70) has multiple biological functions, including protein chaperoning, immune responses, boosting cellular stress tolerance, and participating in the formation and repair of the cytoskeleton. It has been reported that Hsp70 can bind to Apaf-1, c-Jun N-terminal kinase (JNK), and apoptosis-inducing factors to inhibit apoptosis (Beere *et al.* 2000; Park *et al.* 2001; Ravagnan *et al.* 2001). In the hepatocytes of stressed mice, the expression of anti-apoptotic heat shock proteins such as Hsp27, Hsp32, and Hsp70, as well as c-Jun was increased (Vogel *et al.* 2004). In human colon cancer cells, overexpression of Hsp70 has been shown to suppress TNF-α-mediated NF-κB signaling pathway activation by interacting with TNF receptor (TNFR) coupling factor 2 (Dai *et al.* 2010). Therefore, the JNK pathway might modulate TNF-α-mediated apoptosis in colon cancer cells. Furthermore, it has been demonstrated that overexpression of Hsp70 not only alleviated oxidative stress-induced necrosis of muscle cells, but also inhibited apoptosis by preventing the mitochondrial release of Cyt *c* and Smac (Xiao *et al.* 2004). Despite evidence from several studies that Hsp70 displays an anti-apoptotic function, the mechanism is still not clear. Therefore, the objective of this study was to investigate the hypothesis that overexpression of Hsp70 could protect rat hepatocytes from H_2O_2-induced apoptosis and to reveal the mechanism. This study will provide a new insight into the molecular mechanisms of apoptosis inhibition.

Materials and Methods

Cell culture Buffalo rat liver (BRL) cells were purchased from the Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, and cultured in Dulbecco's Modified Eagle's Medium (DMEM)/high-glucose medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin-streptomycin (all from Solarbio, Beijing, China). The Hsp70 overexpressed adenovirus was from our lab (Animal Physiology Lab, Heilongjiang Bayi Agricultural University).

Cell model establishment The working concentration of H_2O_2 was determined by cell viability, oxidative damage, and apoptosis after exposure to different concentrations of H_2O_2 (0, 50, 100, 150, 200, and 250 μmol/L) for 2 h. After determining the working concentration of H_2O_2, in the following experiment, cells were divided into six groups. Cells in groups 1–3 were cultured in normal medium; however, groups 4–6 were cultured in medium with certain concentration (150 μmol/L) of H_2O_2: group 1 (BRL cells), group 2 (BRL cells infected with an empty recombinant adenovirus, Ad-CMV-Null), group 3 (BRL cells infected with an Hsp70-encoding recombinant adenovirus, Ad-CMV-Hsp70), group 4 (BRL cells treated with H_2O_2), group 5 (BRL cells treated with H_2O_2 and infected with the empty recombinant adenovirus), and group 6 (BRL cells treated with H_2O_2 and infected with an Hsp70-encoding recombinant adenovirus).

Evaluation of cell viability BRL cells (1.5×10^3/well) were seeded into 96-well plate, then incubated at 37°C for 48 h. Afterwards, cells were treated with different concentrations of H_2O_2 (H3410, Sigma, St. Loius, MO) according to the experimental requirements. Each group was composed of five replicates. WST-1 (C0036, Beyotime, Jiangsu, China, Cell Proliferation and Cytotoxicity Assay Kit) detection was performed after 2 h of H_2O_2 treatment. This experiment was repeated at least three times.

Assessment of cell oxidative damage Each group of BRL cell was harvested and washed twice with pre-cold PBS. Cells were scraped and transferred into a 1.5-mL centrifuge tube. After centrifugation, the supernatant was discarded and pellet was resuspended in cold saline and sonicated at 40% magnitude for 10 cycles of 5 s on and 30 s off. A total of 450 μL homogenate was used to determine the protein carbonyl concentration (Protein Carbonyl assay kit, A087, Jiancheng, Nanjing, China) and total superoxide dismutase (SOD) activity (Total Superoxide Dismutase Assay Kit with NBT, S0107, Beyotime, Jaingsu, China). This experiment was repeated at least three times.

Flow cytometry analysis Cells (1×10^6) were harvested for apoptosis assessment by Annexin-V/FITC-P cell apoptosis detection kit (C1063, Beyotime) and gently resuspended in 195 μL of Annexin V-FITC binding buffer. Then, 5 μL Annexin V-FITC was added, gently mixed, and incubated at room temperature 15 min in the dark. Subsequently, 5 μL of propidium iodide (PI) staining solution was added and gently

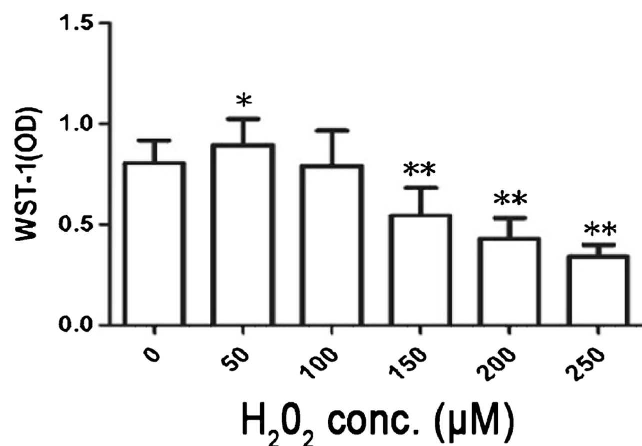

Figure 1. Effect of H_2O_2 on the cell viablity of BRL cells. $^*P < 0.05$,$^{**}P < 0.01$. (*Conc.* concentration).

mixed. The samples were placed in an ice bath in the dark before being examined by flow cytometry. This experiment was repeated three times.

Separation of mitochondria Mitochondria were isolated from cells using the Cell Mitochondria Isolation Kit (C3601, Beyotime) according to the manufacturer's instructions. The lysates were centrifuged at 3000*g* for 5 min, and then, the supernatants were centrifuged at 15,000*g* for 10 min. The supernatant contained the cytosolic fraction. The pellet was dissolved in 0.1% sodium dodecyl sulfate (SDS; L3771, Sigma) and centrifuged at 15,000*g* for 20 min. The supernatant contained the mitochondrial fraction.

Western blot The cells were directly lysed in gel sample buffer, separated under reducing conditions on 12% SDS-polyacrylamide gels, and subjected to immunoblotting using

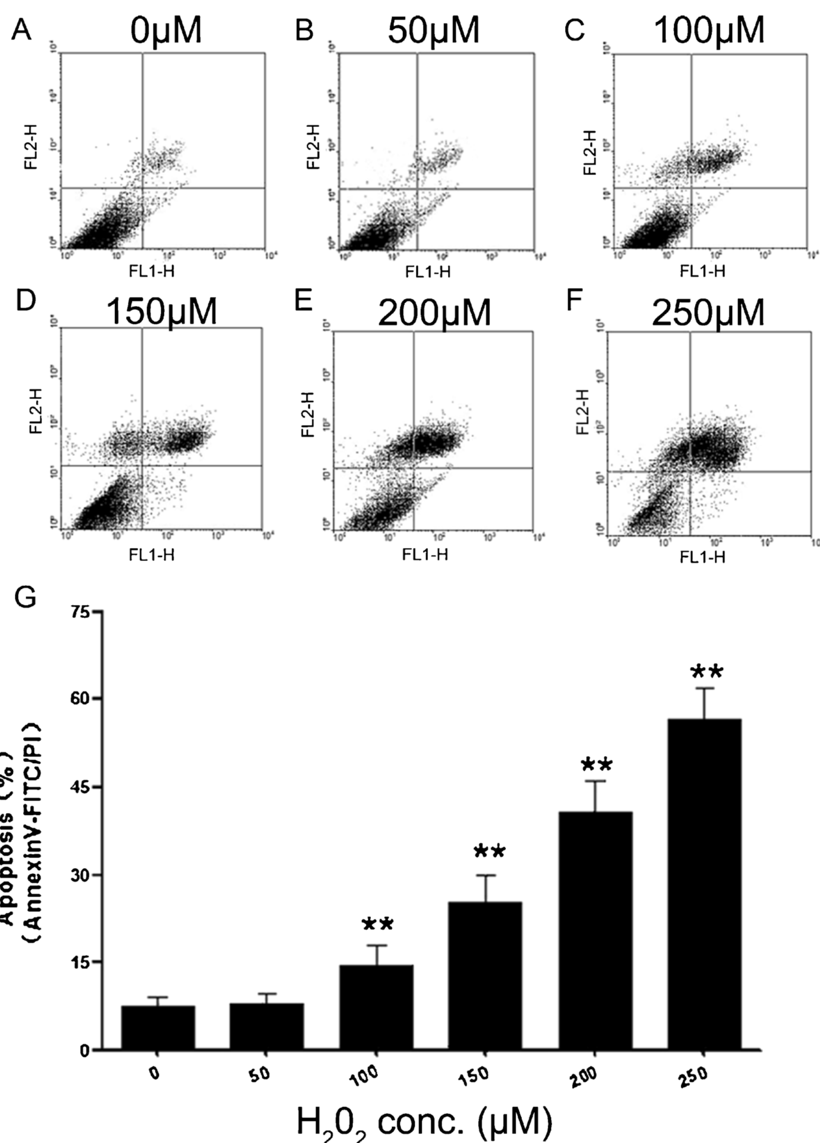

Figure 2. Effect of H_2O_2 on apoptosis of BRL cells. (*A*) Control group. (*B*) 50 μmol/L H_2O_2. (*C*) 100 μmol/L H_2O_2. (*D*) 150 μmol/L H_2O_2. (*E*) 200 μmol/L H_2O_2. (*F*) 250 μmol/L H_2O_2. (*G*) Quantity of flow cytometric analysis. $^{**}P < 0.01$. (*Conc.* concentration).

Table 1 Effect of H_2O_2 on protein carbonyl content and SOD activities of BRL cells.

Group	Protein carbonyl content (nmol/mg prot)	SOD (U/mg prot)
Control	2.01 ± 0.21	44.87 ± 1.85
150 μmol/L H_2O_2 treatment	3.20 ± 0.66*	40.88 ± 1.12*

*$P < 0.05$

primary antibodies against Hsp70 (1:1000), cleaved caspase 8 (1:800), Bcl-2 (1:200), Bax (1:2000), Cyt *c* (1:500), cleaved caspase3 (1:500), and β-actin (1:1000), and detection was made using HRP-conjugated goat anti-mouse IgG (1:10,000). The anti-Hsp70 antibody (ab47455) was purchased from Abcam (Cambridge, MA). The anti-β-actin (AA128-1), anti-cleaved caspase 3 (AC003), and HRP-conjugated goat anti-mouse IgG antibodies (A0216) were purchased from Beyotime. The anti-cleaved caspase 8 (13423-1-AP), anti-Bax (60267-1-LG), and anti-Cyt *c* (10993-1-AP) antibodies were purchased from Proteintech (Chicago, IL). The anti-Bcl-2 (BA0412) antibody was purchased from BOSTER (Wuhan, China). Signals were generated using chemiluminescence, and the blots were imaged using a Bio-Rad gel imaging system (molecular imager). Densitometry values of the bands were calculated. Target protein levels are expressed as ratios of the band density compared with that of β-actin. This experiment was repeated three times at least.

Quantitative RT-PCR Total RNA was isolated from cells with Trizol reagent (T9424, Sigma) and reverse-transcribed with the RT kit (D6110A, Takara, Dalian, China) following the manufacturer's instructions. Quantitative RT-PCRs were carried out in the presence of SYBR Green (QPK-201, Takara) and the reactions were monitored using a Bioer Real-time instrument ((FQD-48A, Hangzhou Bioer Technology Co., Ltd., Zhejiang, China). Reactions were carried out in triplicate. The resulting data were used to calculate the relative quantity using the maximum second derivative method, which was accomplished with the built-in software of the Line-Gene K fluorescence quantitative PCR device. The following primers (5′ to 3′) were used: Hsp70: GCTCGAGTCCTACGCCTCAATA/TCCTGGCACTTGTCCAGCAC; β-actin: TCACCAAC TGGGACG/GCATACAGGGACAACA. This experiment was repeated three times at least.

Statistical analyses The SPSS 19.0 (IBM, New York, NY) was used to perform the relevant statistical analyses. All experimental data were expressed as the means ± standard deviations ($x \pm s$). Univariate analysis of variance and *t* test were used as the statistical methods. $P < 0.05$ was considered statistically significant.

Results

Effect of H_2O_2 on the viability of BRL cells As shown in Fig. 1, cells treated with 50 μmol/L H_2O_2 for 2 h displayed elevated cell viability ($P < 0.05$), whereas cells treated with 100 μmol/L H_2O_2 exhibited a slight reduction in viability, compared with the control group which received no H_2O_2 treatment. Cells treated with over 150 μmol/L H_2O_2 showed a significant decrease in cell viability ($P < 0.01$), with a trend

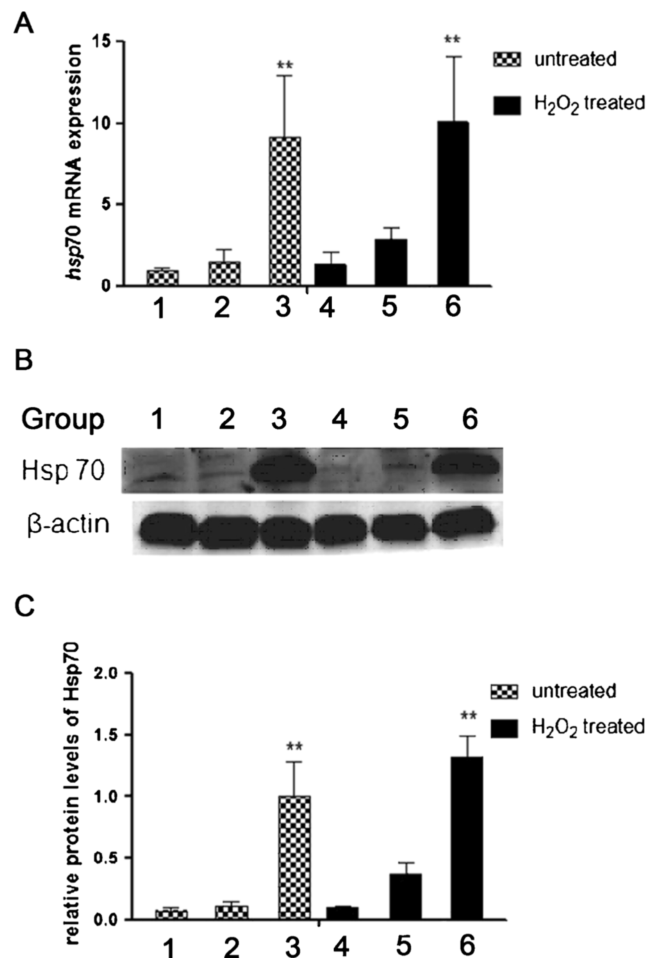

Figure 3. Hsp70 mRNA and protein levels. (*A*) Hsp70 mRNA levels. (*B*) Hsp70 protein levels. (*C*) Hsp70/β-actin ratios determined by densitometry. *Untreated* groups 1, 2, and 3. *H_2O_2 treated* groups 4, 5, and 6. *Control* group 1 and group 4. *Ad-CMV-Null* group 2 and group 5. *Ad-CMV-Hsp70* group 3 and group 6. **$P < 0.01$.

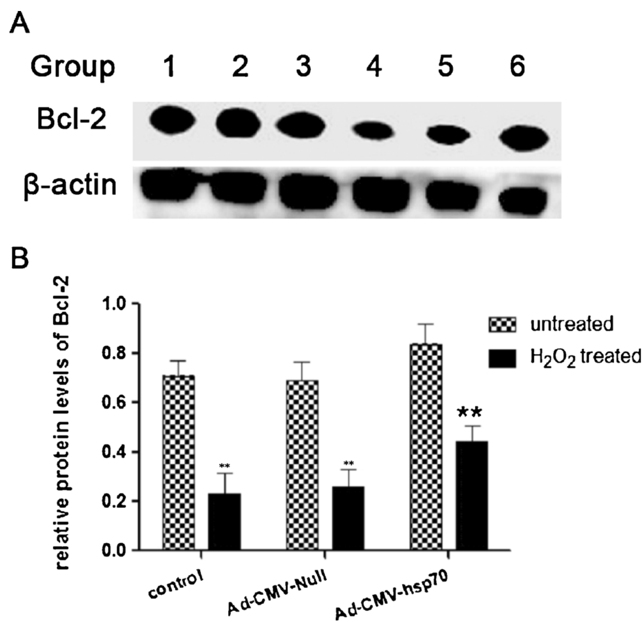

Figure 4. Effect of Hsp70 overexpression on Bcl-2 protein levels. (*A*) Bcl-2 protein expression measured by western blotting. (*B*) Bcl-2/β-actin ratios determined by densitometry. *Untreated* groups 1, 2, and 3. *H_2O_2 treated* groups 4, 5, and 6. *Control* group 1 and group 4. *Ad-CMV-Null* group 2 and group 5. *Ad-CMV-Hsp70* group 3 and group 6. $^{**}P<0.01$.

of higher H_2O_2 concentrations correlating with lower cell viabilities.

Effect of H_2O_2 on the apoptosis of BRL cells As shown in Fig. 2, apoptosis occurred at a very low frequency in the control group. However, after 2 h treatment of H_2O_2, the percentage of apoptotic cells increased and positively correlated with H_2O_2 concentrations. Specifically, the percentage of apoptotic cells for each group was as follows: 50 μmol/L H_2O_2, 8.09 ± 0.57%; 100 μmol/L H_2O_2, 14.37 ± 0.52%; 150 μmol/L H_2O_2, 25.41 ± 0.54%; 200 μmol/L H_2O_2, 40.74 ± 0.45%; and 250 μmol/L H_2O_2, 56.51 ± 0.54%. In addition, H_2O_2 also caused cell death. The percentage of dead cells for each group was as follows: 100 μmol/L H_2O_2, 4.85 ± 0.55%; 150 μmol/L H_2O_2, 7.89 ± 0.61%; 200 μmol/L H_2O_2, 16.17 ± 0.63%; and 250 μmol/L, 15.94 ± 0.65%, respectively. Hence, we chose 150 μmol/L H_2O_2 treatment for the following experiments.

Effect of H_2O_2 on the protein carbonyl content and SOD activity of BRL cells As shown in Table 1, cells treated with 150 μmol/L H_2O_2 for 2 h displayed increased protein carbonyl content and decreased total SOD activity, and both parameters were significantly different from the corresponding values of the control group ($P<0.05$). This revealed that exposure to 150 μmol/L of H_2O_2 resulted in oxidative stress to cells.

Overexpression of Hsp70 in BRL cells As shown in Fig. 3A, cells infected with an adenovirus expressing Hsp70 showed a significantly higher level of Hsp70 mRNA than that of the control groups ($P<0.01$). Likewise, cells infected with an adenovirus expressing Hsp70 showed a significantly higher abundance of Hsp70 protein levels than that of the control groups ($P<0.01$) (Fig. 3B). Hsp70/β-actin ratios determined by densitometry are shown in Fig. 3C.

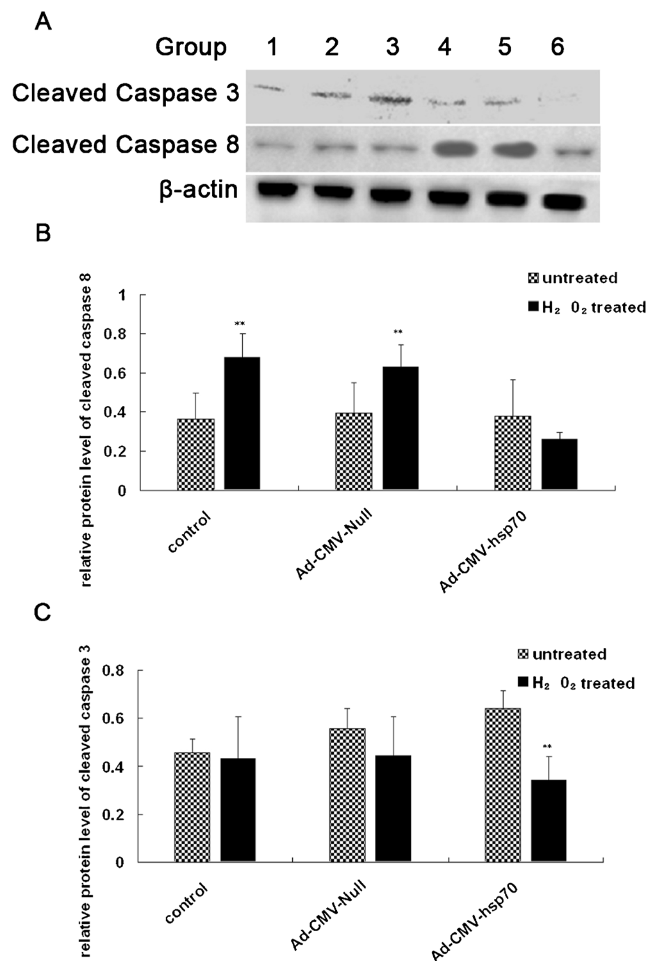

Figure 5. Effect of Hsp70 overexpression on cleaved caspase 3 and cleaved caspase 8 protein levels. *Untreated* groups 1, 2, and 3. *H_2O_2 treated* groups 4, 5, and 6. *Control* group 1 and group 4. *Ad-CMV-Null* group 2 and group5. Ad-CMV-Hsp70: group 3 and group 6. $^{**}P<0.01$.

Effect of Hsp70 overexpression on Bcl-2, caspase 3, caspase 8, Cyt *c*, and Bax levels in BRL cells As shown in Fig. 4, cells treated with H_2O_2 (groups 4, 5) displayed significantly lower protein levels of Bcl-2 ($P<0.01$) than their equivalent group without H_2O_2 treatment (groups 1, 2). However, in group 6, where Hsp70 was overexpressed, Bcl-2 levels were not significantly decreased after H_2O_2 treatment compared to the untreated cells (group 3) ($P>0.05$). Similarly, cleaved caspase 3 (Fig. 5) displayed lower levels in the H_2O_2 treatment groups (4, 5) compared to the untreated groups (1, 2). Whereas, caspase 8 (Fig. 5) displayed significantly higher levels in the H_2O_2 treatment groups (4, 5) compared to the

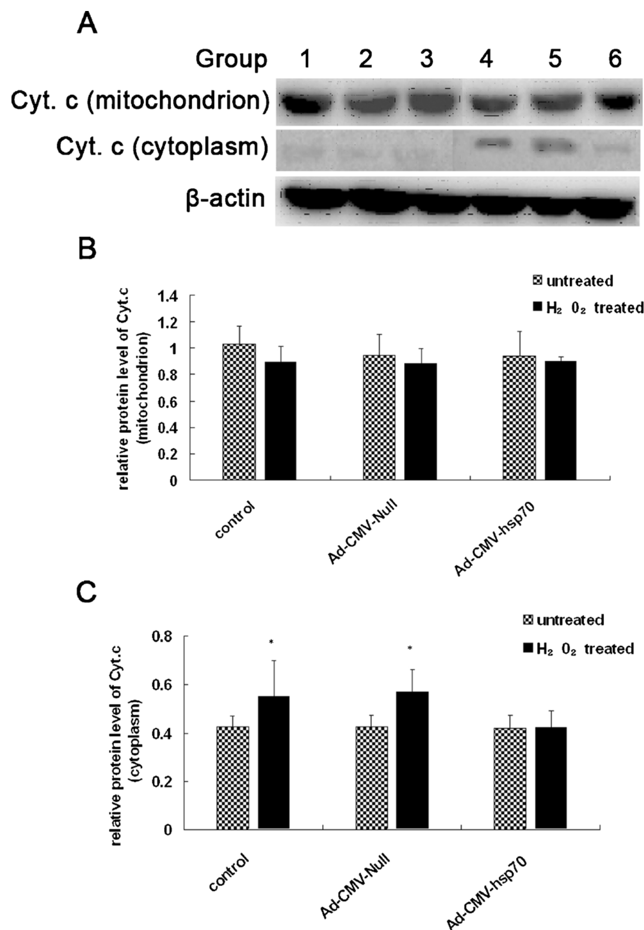

Figure 6. Effect of Hsp70 overexpression on Cyt *c* protein levels. *Untreated* groups 1, 2, and 3. *H_2O_2 treated* groups 4, 5, and 6. *Control* group 1 and group 4. *Ad-CMV-Null* group 2 and group 5. *Ad-CMV-Hsp70* group 3 and group 6. $^*P<0.05$.

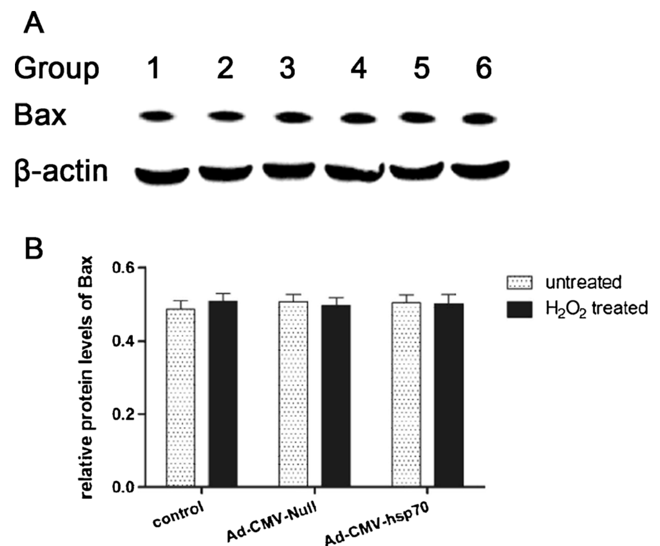

Figure 7. Effect of Hsp70 overexpression on Bax protein levels. (*A*) Bax protein expression measured by western blotting. (*B*) Bax/β-actin ratios determined by densitometry are shown above the western blots. *Untreated* groups 1, 2, and 3. *H_2O_2 treated* groups 4, 5, and 6. *Control* group 1 and group 4. *Ad-CMV-Null* group 2 and group 5. *Ad-CMV-Hsp70* group 3 and group 6.

untreated groups (1, 2). However, compared to the untreated cells, those infected with the Hsp70 overexpressing adenovirus (group 6) had a significant reduction in cleaved caspase 3 and caspase 8 ($P<0.01$), even after H_2O_2 treatment (group 3).

As shown in Fig. 6, cells treated with H_2O_2 (groups 4, 5) exhibited a significant release of Cyt *c* from the mitochondria into the cytoplasm, compared with cells without H_2O_2 exposure (groups 1, 2, 3). However, in the Hsp70 overexpressed group (group 6), the amount of Cyt *c* released was significantly reduced ($P<0.05$). Nevertheless, no difference was found in Bax protein levels (Fig. 7) in all the groups of BRL cells.

Effect of Hsp70 overexpression on H_2O_2-induced apoptosis in BRL cells As shown in Fig. 8, cells treated with H_2O_2 (Fig. 8B, D, F, groups 4, 5, 6) all exhibited a higher percentage of apoptotic cells than the untreated cells (Fig. 8A, C, E, groups 1, 2, 3). However, cells infected by the adenovirus overexpressing Hsp70 (Fig. 8F, group 6) showed a markedly lower percentage of apoptotic cells than the control groups (Fig. 8B, D, group 4, 5) ($P<0.01$).

Discussion

Oxidative stress can cause apoptosis through multiple pathways, such as the mitochondria, the death receptor, or ER stress (Huang *et al.* 2003; Singh and Singh 2008; Li *et al.* 2010). Alternatively, oxidative stress also activates the mitogen-activated protein kinase (MAPK) pathway and the caspase cascade to induce apoptosis (Chen *et al.* 2001; Zhuang *et al.* 2007). The expression of Hsps is closely linked to cell growth and differentiation. These proteins play pivotal regulatory roles in the aforementioned apoptosis pathways. Hence, in this study, 150 μmol/L H_2O_2 was used to induce apoptosis of rat BRL cells to explore the mechanisms how Hsp70 modulates H_2O_2-induced apoptosis.

A large body of evidence suggests that the Bcl-2 family members play important regulatory roles in the mitochondrial pathway of apoptosis. Bcl-2 is a critical anti-apoptotic protein that stabilizes the permeability of the mitochondrial membrane (Chen *et al.* 2001). In the mitochondrial pathway, Cyt *c* is the key mediator and is released from the mitochondria into the cytoplasm when cells are under stress. Our results revealed that after cells are exposed to H_2O_2, the amount of Cyt *c* released from the mitochondria into the cytoplasm was significantly increased, and this was accompanied with reduced expression of the Bcl-2 protein. Correspondingly, cells displayed apoptotic damage. Although Bax levels were not significantly changed, the ratio of apoptosis-related proteins Bcl-2/Bax was changed, as we expected. This indicates that

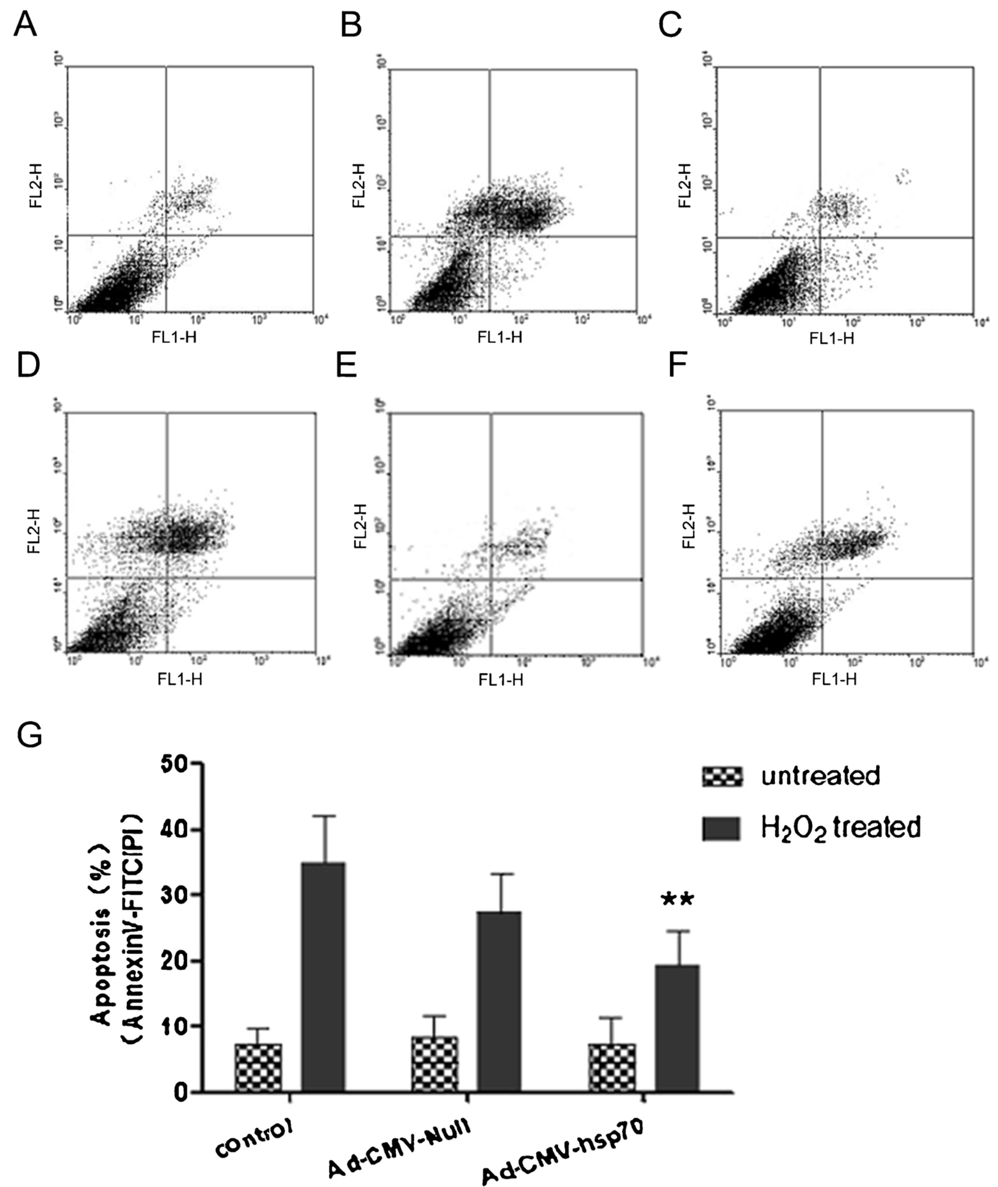

Figure 8. Effect of Hsp70 overexpression on H2O2-induced apoptosis in BRL cells. (*A*) Group 1. (*B*) Group 4. (*C*) Group 2. (*D*) Group 5. (*E*) Group 3. (*F*) Group 6. (*G*) Quantity of flow cytometric analysis. *Control* A and B. *Ad-CMV-Null* C and D. *Ad-CMV-Hsp70* E and F. $^{**}P < 0.01$.

oxidative stress may induce apoptosis by multiple routes, such as affecting mitochondrial membrane permeability, promoting the release of Cyt *c* into the cytoplasm, and regulating Bcl-2 levels.

Caspase 3 and caspase 8 are the main executors of apoptosis, and their activation is a central event in apoptosis. Our results indicate that H_2O_2-induced apoptosis of rat BRL cells was stimulated by the activation of caspase 3 and caspase 8, which is consistent with a previous report (Zhu *et al.* 2006). Our data also reveals that cells infected with an adenovirus overexpressing Hsp70 exhibited a decrease in the activation of caspase 3 and caspase 8, resulting in reduced apoptosis after exposure to H_2O_2. Together, this indicates that Hsp70 may regulate the activation of caspase 3 and caspase 8 to protect cells from apoptosis.

Conclusions

In this study, cellular oxidative stress was induced by H_2O_2, which regulates apoptosis via multiple pathways. Overexpression of Hsp70 inhibits the activation of caspase 3 and caspase 8, and also modulates the release of Cyt *c* into the cytoplasm. These events enhance the expression of the anti-apoptotic protein Bcl-2 and suppress the activation of caspase 3. As a consequence, BRL cells are protected from apoptosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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